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March 26, 1999

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MR 21249

Document Processing Center (7407)
Attention: Section 8(e) Coordinator
Office of Pollution Prevention & Toxics
U.S. Environmental Protection Agency
401 M Street, SW
Washington, DC 20460-0001

Dear Sir/Madam:

N,N-Dimethylacetamide, CAS 127-19-5 and
N-Monomethylacetamide, CAS 79-16-3

We received the attached prepublication copy of a paper on the above-referenced chemicals from our European subsidiary. We understand that the final version has been accepted for publication in Toxicology In Vitro published by Elsevier Science. Publication date is unknown.

The work that led to the results being reported was not sponsored or supported by DuPont. However, as manufacturers of N,N-Dimethylacetamide and in recognition of EPA's June 1991 guidance on the reportability of toxicological data under TSCA §8(e), we are submitting the paper on a For Your Information basis. (N-Monomethylacetamide is the metabolite of N,N-Dimethylacetamide).

Since we have no further details on the study or the results, EPA should contact the author if additional information is needed.

I may be contacted at 609/540-4576 if there are any questions relating to this communication.

Yours truly,

K D Dastur

Kavsy D. Dastur
Manager, Product Toxicology &
Chemical Regulations

/mn
Attachment
Certified Mail
P 203 970 493

Title: *In Vitro* Embryotoxicity Study of N-N-dimethylacetamide and its main Metabolite N-monomethylacetamide.

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Running title: Dimethylacetamide embryotoxicity *in vitro*.

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ABSTRACT

N-N-dimethylacetamide (DMAC) is a widely used industrial solvent. Previous teratological studies *in vivo* reported discording results. Using the postimplantation rat whole embryo culture (WEC) method, the direct embryotoxic effects of DMAC and its main metabolite (N-monomethylacetamide, MMAC) have been investigated in the present work. Both chemicals showed specific embryotoxic effects at similar concentration levels.

The no-observed-effect level (NOEL) was 0.85 mM. Macroscopically, the main target organs were somites, brain and branchial bars. Histological examination revealed an increase in cell death at the effective concentrations on the neuroepithelium and branchial bars mesenchyme. The results of this work, together with those obtained in *in vivo* studies, suggest that the exposure limits in workplaces could be inappropriate for the safety of fertile women.

Abbreviations: DMAC: N-N-dimethylacetamide; MMAC: N-monomethylacetamide; WEC: whole embryo culture; NOEL: no-observed-effect-level; VYS: visceral yolk sac.

Keywords: N-N-dimethylacetamide, N-monomethylacetamide, embryotoxicity, whole embryo culture.

INTRODUCTION

N-N-Dimethylacetamide (DMAC) is an industrial solvent widely used in the manufacture of coatings, fibres, foils and lacquers. Potential human exposure is through inhalation of vapours or skin contact with the liquid substance in the workplace. The occupational exposure limit (TLV) worldwide is accepted at the value of 10 ppm in air, assuming that it may be also safe for women in fertile age.

Several studies on potential developmental toxicity of DMAC have been performed on different animal species and by different routes of administration, with equivocal results. In a one generation reproduction study, Ferenz and Kennedy (1986) exposed male and female Sprague-Dawley rats to 0, 30, 100 or 300 ppm DMAC, by inhalation, 6hr/day, 5 days/week during premating, mating, gestation and postnatal period. No adverse effects were seen on mating, fertility, gestation, parturition, litter size and postnatal survival of pups. The only effect observed was reduced pup weight at the top dose.

Solomon *et al.* (1991) exposed pregnant Sprague-Dawley rats to 0, 30, 100 or 300 ppm DMAC, by inhalation, for 6hr/day from days 6 to 15 of pregnancy. Reduced body weight gain was observed in females of the highest dose group. Fetal weight was reduced in this group, but no other signs of embryotoxicity were seen.

Limited developmental studies following dermal application have been reported by Stula and Krauss (1977). No clear effects on resorptions were observed after applications of 600 mg/Kg body weight on days 9, 10 and 11; 11 and 12; or 12 and 13 of gestation, but evidence of reduction of fetal body weight was reported. Rats dosed with 1200 mg/Kg body weight on days 9; 10 and 11; or 11 and 12 showed embryomortality and severe reduction in fetal weights. One litter from a dam treated on days 10 and 11 had three foetuses with encephalocele.

No embryotoxic effects were observed in five rabbits treated dermally with 200 mg/Kg/day from days 8 to 16 of pregnancy. Merkle and Zeller (1980) administered pregnant Russian rabbits with 0, 94, 282, 470 mg/Kg/day DMAC, by gavage, on days 6-18 of pregnancy. The highest dose produced severe maternotoxicity and all implantations were resorbed. Maternotoxicity was also evident at mid dose associated with reduced numbers of live foetuses and reduced fetal weight. Five foetuses had malformations (cleft palate, fused ribs and microphthalmia). The lowest dose caused slight maternotoxicity but no adverse effects on embryo-foetal development.

In a teratology study on COBS CD rats, Johannsen *et al.* (1987) administered, by gavage, 0, 65, 160 or 400 mg/Kg/day DMAC on days 6-19 of gestation. A clear reduction in body weight gain was observed at the top dose. There was a significant increase in resorptions, reduced fetal weight and a large number of foetuses with malformations (49/292). The majority of the malformations were at the level of the cardiovascular system. No embryotoxic effects were observed at the mid and low dose levels. On the basis of the results obtained in these studies it is difficult to assess the embryotoxic and teratogenic potential of DMAC and discriminate between the direct effects of DMAC on the embryos and the effects mediated by maternal toxicity.

The purpose of this study was to evaluate the direct embryotoxic potential of DMAC on rat embryos. As the main metabolite of DMAC both in rats and in humans has been identified as N-Monomethylacetamide (MMAC), we also tested this substance in order to verify if the embryotoxic effects were due to the parental molecule or its metabolite. For that purpose we used postimplantation rat whole embryo culture (WEC) which avoids the potentially confounding influence of maternal factors.

MATERIALS AND METHODS

Crl:CD Sprague-Dawley rats (Charles River, Calco, Italy) were housed in standard conditions (T=21±1°C; relative humidity=50±5%; light-dark cycle 6:18) with food (Italiana Mangimi) and tap water ad libitum. After one week of acclimatisation, virgin females were mated overnight with males of the same strain. The morning of positive smear was considered as day 0 of gestation. 9.5 day-old embryos (embryonic stage 1-3 somites) were explanted from pregnant rats and cultured for 48 hr into 20 ml culture bottles, inserted in a thermostatic (38°C) roller apparatus (30 rpm).

The culture medium was heat-inactivated rat serum supplemented with an antibiotic sterile solution (final concentration: 100 IU/ml penicillin and 100 µg/ml streptomycin), equilibrated with gas mixtures containing increasing O_2 concentrations, according to the method described by Giavini *et al.* (1992).

DMAC (Sigma) and MMAC (Fluka) were dissolved in sterile saline and added (100 µl/5 ml rat serum/ 5 embryos) to the culture medium of treated groups in order to obtain the final concentrations of 0.85, 1.75, 3.5, 5 mM for both chemicals. After 48 hr of culture the embryos were morphologically examined and scored according to Brown and Fabro (1981). The visceral yolk sac (VYS) diameter, crown-rump length, head length and somite number were also recorded.

A few embryos were processed for histological examination: fixed in 4% formaldehyde, embedded in Technovit 7100 and stained with hematoxilin and eosin. The rest of the embryos and VYS were evaluated for total protein (Bradford, 1976) and DNA (Labarca and Paigen, 1980) content.

Statistical analyses were performed using one-way ANOVA with Tukey's multiple comparisons and the chi-square test. The level of significance was set at $p<0.05$.

RESULTS

Exposure to DMAC or MMAC 3.5 and 5mM produced concentration-related effects on growth and development: the yolk sac diameter (DMAC), crown-rump length, somite number and total score were reduced (Tab. 1). Total protein content was significantly reduced in DMAC 3.5 and 5 mM and in MMAC 5mM; DNA content was reduced in DMAC and MMAC 5mM; the protein/DNA ratio was increased in DMAC and MMAC 5mM (Tab. 2). The effects on VYS biochemical parameters resulted in a significant reduction in protein/DNA in 3.5 and 5mM MMAC (Tab. 2).

Dysmorphogenic effects of DMAC and MMAC (Fig. 1; Fig. 2) were apparent at 1.7, 3.5 and 5 mM. The incidence and severity of the effects were concentration-dependent. The most prominent and specific effect induced by DMAC and MMAC was a schisis of the anterior part of the brain.

A high frequency of embryos with severe reduction of first and second branchial bars was present in the group exposed to DMAC 5mM, but sporadic cases were also observed at 3.5 mM DMAC and 3.5 and 5 mM MMAC. Abnormal somites were observed in embryos exposed to DMAC 3.5 and 5mM, with a higher frequency than in the control group. Other kinds of abnormalities were randomly observed in all groups and were not considered substance-dependent.

Histological examination (Fig. 2) revealed a concentration-dependent increase in foci of cell death in embryos exposed to DMAC and MMAC 1.7 mM and higher. The most frequent tissues affected were the mesenchyme of maxillary processes, branchial bars and the neuroepithelium.

DISCUSSION

Our results show that DMAC and MMAC can directly affect the development of rat embryos grown *in vitro*, producing concentration-related growth retardation and dysmorphogenesis. The solvent and its main metabolite caused comparable characteristic alterations at the level of the forebrain which was widely open and/or with histological signs of cell death. At the top concentration morphological and at all effective concentrations histological severe alterations at the level of first and second branchial bars were also observed. Our results confirm the teratogenic potential of DMAC and also show that the main metabolite possesses similar teratogenic potential. As *in vivo* DMAC is rapidly converted in MMAC (after a multiple 6 hr exposure to 150 ppm, in rats the half-life of DMAC was 0.6 hr and DMAC was undetectable in plasma after 12hr, whereas the half life of MMAC was 2.2 hr and a significant amount of MMAC was still present in plasma after 24 hr) (Hundley et al, 1994), it is impossible to determine if the embryotoxic effects observed in *in vivo* experiments are induced by DMAC or by the metabolite MMAC.

It is difficult to compare the dysmorphogenic effects obtained in this study to those obtained in *in vivo* studies. The main teratogenic effect observed in rat foetuses after maternal exposure to DMAC by gavage (Johannsen *et al.*, 1987) was at the level of the cardiovascular system. No malformations have been reported at the level of the brain and only few cases of micrognathia have been signalled.

A few cases of encephalocele have been reported by Stula and Krauss (1977) in rat foetuses after maternal exposure to very high doses of DMAC by dermal route. For these reasons it is quite difficult to interpret our results. The absence in our study of evident signs of cardiovascular malformations observed by other authors (common truncus arteriosus, no ductus arteriosus, interventricular septal defects, retroesophageal aorta) may be due to the fact that the embryos at the observation stage at the end of the culture are too young and only very

severe alterations of the heart are visible (e.g. dextrocardia), while other kinds of cardiovascular malformations are apparent only in later stages.

The interpretation of the forebrain abnormalities is also not clear: it could be a real malformation or a sign of delayed closure of brain folds. This interpretation is supported by the observation that the administration of Valproic Acid (Menegola *et al.*, 1996) or Retinyl Palmitate (Piersma *et al.*, 1995) results in a high frequency of embryos with open brain folds, when observed at midgestation, but no cerebral malformations in foetuses at term, suggesting that in some cases delayed cranial neural tube closure may be completed later in gestation. These limits are intrinsic to the method used in *in vitro* experiments; however, the forebrain neuroepithelium was clearly shown to be the specific target organ of both DMAC and MMAC, suggesting a possible role of these chemicals in morphological or functional brain damage and the need for more thorough *in vivo* investigations for neurotoxicological effects.

The no effect concentration for DMAC and MMAC has been considered 0.85 mM, also on the basis of the results obtained from histological examination.

Unfortunately no data of DMAC pharmacokinetics are available in the literature for oral administration, so a comparison with the results obtained by Johannsen *et al.* (1987) is not possible. As the dose response relationship in that study was very steep, it is possible that very high peak blood concentrations, achievable by bolus gavage administration are necessary to produce the observed teratogenic effects.

The results of the present work agree with those obtained by Solomon *et al.* (1991) in their experiment by inhalation, in which the no effect level for embryotoxicity was 100 ppm; as the plasma concentration of about 1 mM DMAC has been observed in rats exposed by inhalation for 6 hr to 150 ppm DMAC (Hundley *et al.*, 1994), the plasma concentration after exposure to 100 ppm in air may be similar to the no effect concentration observed in our study.

As the *in vivo* kinetics after inhalation exposure is not very different from the *in vitro* situation (the plasma C_{max} is rapidly reached and maintains constant values until the end of exposure), the no effect concentrations obtained in our study could be compared to plasma concentrations observed in rats after inhalation exposure and used for a tentative correlation of safety exposure in humans. The TLV in workplace is 10 ppm, corresponding to a dose level of about 5.4 mg/Kg/day, assuming that a worker breathes 10 m^3 of air in 8 hr. The dose of 100 ppm in air corresponds to 360 mg/m^3. For a rat of 300 g an exposure of 6 hr means a dose of 54 mg/Kg/day , i.e., ten times the accepted TLV. It may be a matter of discussion if this TLV value is enough for adequate protection of female workers.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. - Specific dysmorphogenic effects induced by DMAA and MMAA after exposure *in vitro*.

* $p<0.05$ vs. CONTROL

** $p<0.01$ vs. CONTROL

Figure 2. - a) Control; b-c) MMAC 5 mM. Embryo affected by a clear developmental delay. The schisis at the level of the anterior and middle part of the encephalon is well recognisable (arrowheads); d-e) Histological section of the neuroepithelium of a control embryo (d) and of an embryo exposed to MMAA 3.5mM (e), in which clear and severe signs of cellular death are recognisable (arrows).

Table 1: Embryonic and Visceral Yolk Sac morphometrical parameters (M±SD).

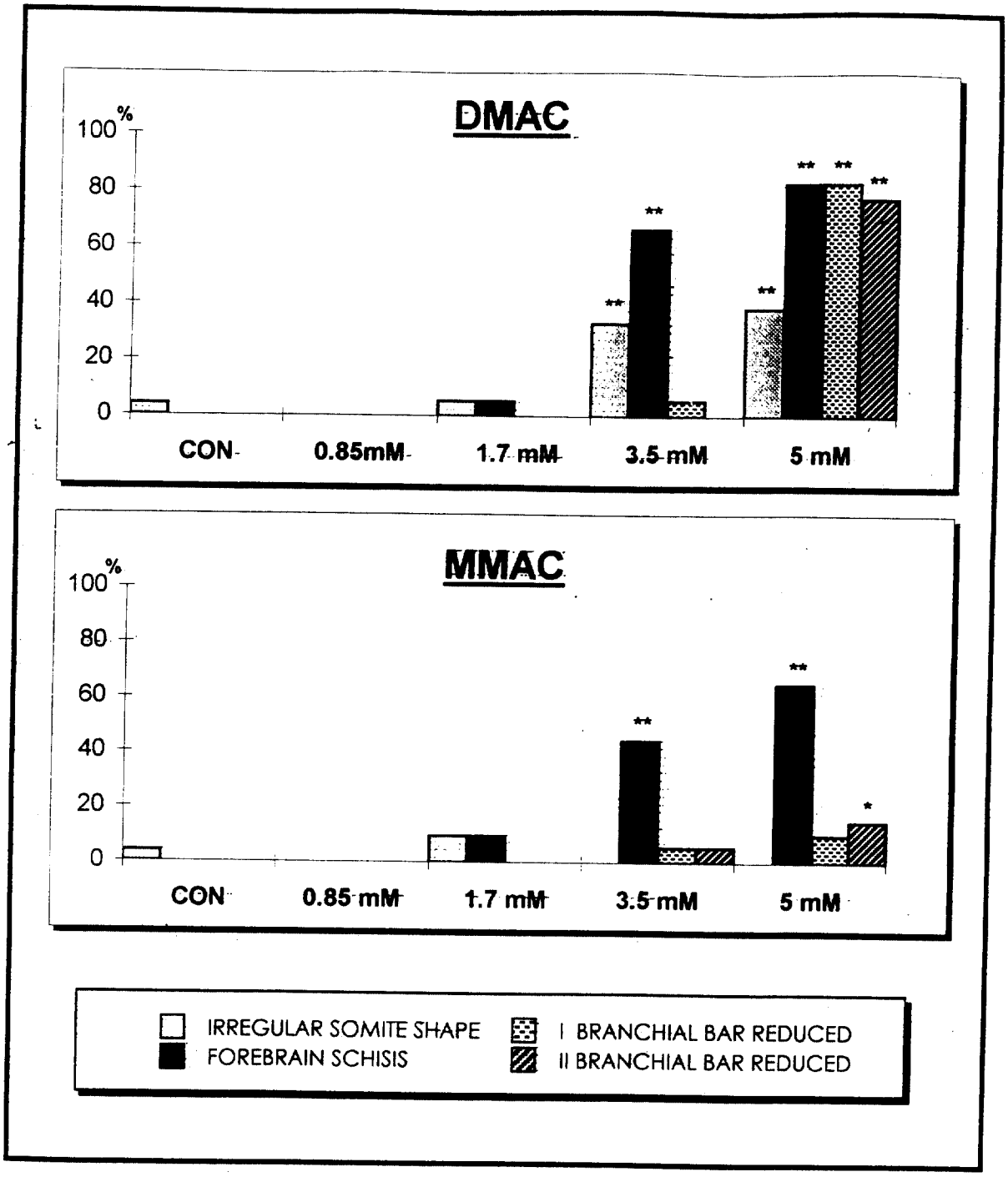
	GR I **CON** *(N=25)*	GR II **0.85 mM** *(N=18)*	GR III **1.7 mM** *(N=19)*	GR IV **3.5mM** *(N=18)*	GR V **5mM** *(N=17)*	GR VI **0.85mM** *(N=19)*	GR VII **1.7mM** *(N=22)*	GR VIII **3.5 mM** *(N=15)*	GR IX **5 mM** *(N=20)*
VYS DIAMETER (mm)	4.35 ± 0.38 *(N=24)*	4.25 ± 0.37 *(N=17)*	4.31 ± 0.21	4.14 ± 0.19 *(N=17)*	aa 4.00 ± 0.31	aa 3.99 ± 0.32	4.28 ± 0.23 *(N=19)*	4.18 ± 0.23	a 4.03 ± 0.25
CROWN-RUMP LENGTH (mm)	3.87 ± 0.22	3.73 ± 0.33	3.73 ± 0.13	aa 3.57 ± 0.19	aa 3.04 ± 0.22 *(N=14)*	3.70 ± 0.18	3.75 ± 0.19	3.70 ± 0.18	aa 3.46 ± 0.26 *(N=18)*
HEAD LENGTH (mm)	1.84 ± 0.13	1.74 ± 0.18	1.73 ± 0.09 *(N=18)*	1.67 ± 0.11 *(N=6)*	N.D.	1.73 ± 0.11	1.75 ± 0.09 *(N=20)*	1.71 ± 0.09 *(N=8)*	aa 1.51 ± 0.09 *(N=10)*
SOMITE NUMBER	25.04 ± 1.06	24.22 ± 1.70	24.21 ± 1.36	23.94 ± 0.80	aa 21.87 ± 2.00 *(N=15)*	24.11 ± 1.29	24.41 ± 0.85	24.31 ± 1.01	aa 23.45 ± 1.50
TOTAL SCORE	39.48 ± 0.92	39.00 ± 1.08	39.21 ± 1.08	aa 37.11 ± 1.60	aa 33.20 ± 1.70 *(N=15)*	39.11 ± 1.24	39.00 ± 1.38	a 38.00 ± 1.26	aa 36.25 ± 1.62

a $p<0.05$ vs control group
aa $p<0.01$ vs control group
ND = Not Detectable

Table 2: Embryonic and Visceral Yolk Sac biochemical parameters (M±SD).

	GR I **CON** *(N=19)*	GR II **0.85 mM** *(N=14)*	GR III **1.7 mM** *(N=15)*	GR IV **3.5mM** *(N=14)*	GR V **5mM** *(N=14)*	GR VI **0.85mM** *(N=15)*	GR VII **1.7mM** *(N=17)*	GR VIII **3.5 mM** *(N=14)*	GR IX **5 mM** *(N=16)*
embryo									
PROTEIN (µg)	188.10 ± 29.88	175.72 ± 37.21	179.96 ± 21.62	a 159.99 ± 17.64	aa 101.66 ± 17.42	174.74 ± 21.72 *(N=14)*	185.06 ± 16.19	176.90 ± 19.29	aa 153.22 ± 19.95
DNA (µg)	15.42 ± 3.58 *(N=18)*	13.67 ± 3.66	14.73 ± 3.44	12.56 ± 2.98	aa 6.75 ± 1.24	13.12 ± 1.98 *(N=14)*	15.45 ± 2.22	14.45 ± 2.45	aa 11.27 ± 2.11
PROTEIN/DNA (µg/µg)	12.49 ± 1.46 *(N=18)*	13.09 ± 1.74	12.63 ± 2.20	13.24 ± 2.48	aa 15.22 ± 1.99	13.49 ± 1.99 *(N=14)*	12.10 ± 1.25	12.47 ± 1.92	13.83 ± 1.96
YYS									
PROTEIN (µg)	112.30 ± 16.13	110.66 ± 32.72	109.52 ± 10.76	94.23 ± 16.69	95.40 ± 14.19	98.49 ± 19.50	101.24 ± 17.67 *(N=16)*	97.68 ± 19.91 *(N=13)*	91.74 ± 13.81
DNA (µg)	5.41 ± 0.83	5.80 ± 1.57	4.62 ± 1.11	4.28 ± 0.67	4.51 ± 0.75	5.22 ± 1.02	5.59 ± 0.75	5.79 ± 1.04	5.76 ± 0.84 *(N=15)*
PROTEIN/DNA (µg/µg)	21.14 ± 3.97	19.34 ± 3.96	24.70 ± 5.09	22.18 ± 3.58	21.37 ± 2.82	18.87 ± 1.19	18.10 ± 2.91 *(N=16)*	a 16.77 ± 2.01 *(N=13)*	aa 16.15 ± 2.38 *(N=15)*

a $p<0.05$ vs control group
aa $p<0.01$ vs control group


DMAC
100%
80
60
40
20
0
**
CON
0.85mM
1.7 mM
3.5 mM
5 mM
MMAC
100%
80
60
40
20
0
**
*
CON
0.85 mM
1.7 mM
3.5 mM
5 mM
IRREGULAR SOMITE SHAPE
FOREBRAIN SCHISIS
I BRANCHIAL BAR REDUCED
II BRANCHIAL BAR REDUCED